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TUBERCULOSIS

PROTOCOL BOOK

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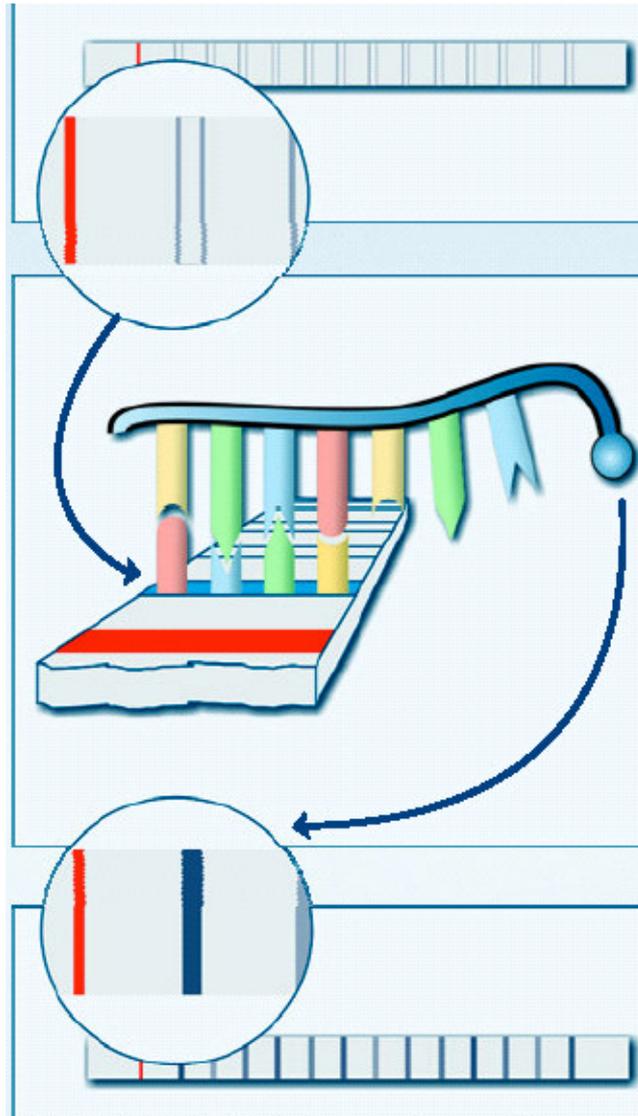
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GenoType[®] (Hain Lifescience)

Adapted from notice for use <http://hain-lifescience.de>



Principle

GenoType® is a Line Probe Assay for identification of the *M. tuberculosis* complex from pulmonary clinical specimens or cultivated samples and its resistance to Rifampicin and/or Isoniazid (GenoType® MTBDR*plus* kit) and Fluoroquinolones and Aminoglycosides/Cyclic Peptides (GenoType® MTBDR*s/* ver 2.0 kit)(1). The whole procedure includes DNA extraction (GenoLyse kit is provided) from pulmonary smear positive or negative clinical specimens or cultivated samples, a multiplex amplification with biotinilated primers (the Taq DNA polymerase is provided) and reverse hybridization.

1. DNA Preparation

The DNA sample should be prepared in a separated area « grey room » (see « Amplification set-up »).

1. Material

- 1.5 mL screw cap tube
- 1000µl, 200 µl, 100µl, 20µl and 2 µl pipettes
- Disposable sterile pipette tips with filter
- 1µl disposable sterile loops (for solid medium culture)
- Container for infectious clinical waste
- Dry bath
- Micro-centrifuge with aerosol tight rotor
- GenoLyse kit (**REF** 12 tests No. 51612, 96 tests No. 51610)
- GenoType® MTBDR*plus* kit (REF 12 tests No. 304A, 96 tests No. 30496A)
- GenoType® MTBDR*s/* kit VER 2.0 (REF 12 tests No. 317A, 96 tests No. 31796A)

2. DNA extraction

a) When using patient specimens

- Transfer 500µl of decontaminated sample material into a labeled 1.5 ml screw cap tube
- Centrifuge for 15 min at 10,000xg in a standard tabletop centrifuge with aerosol tight rotor.
- Discard supernatant and re-suspend pellet in 100µl Lysis Buffer (A-LYS) by vortexing

b) When using bacteria grown in liquid media,

- Transfer 1 mL of liquid medium into a labeled 1.5 ml screw cap tube
- Centrifuge for 15 min at 10000xg in a standard tabletop centrifuge with aerosol tight rotor.

- Discard supernatant and resuspend pellet in 100µl Lysis Buffer (A-LYS) by vortexing

c) When using bacteria grown on solid medium,

- Collect bacteria with an inoculation loop and suspend in 100 µl of Lysis Buffer (A-LYS)
- Vortex

- 1) Incubate sample for 5 min at 95°C in a water or dry bath
- 2) Briefly spin down
- 3) Add 100µl Neutralization Buffer (A-NB) and vortex sample for 5 sec.
- 4) Spin down for 5 min at full speed in a tabletop centrifuge with aerosol tight rotor
- 5) Directly use 5µl of the supernatant for PCR. In case the DNA solution is to be stored for an extended period of time, transfer supernatant to a new tube

2. Amplification

Carefully read the procedure « Amplification set up » and rigorously follow it to avoid contaminations

1. Mix preparation

Prepare the amplification mix (45µl) in a DNA-free « white » room.

- MTBDR*plus* version 2.0 :
Amplification Mix A (AM-A) (150µl) store at +4°C
Amplification Mix B (AM-B) (530µl) store at -20°C
- In each tube :
 - 10µl Amplification Mix A (AM-A)
 - +35µl Amplification Mix B (AM-B)
 - Mix well

2. Addition of DNA

The DNA sample should be added in a separated area « grey room ».

- Add 5µl DNA solution
- Briefly spin down

3. Amplification profile

Clinical specimens

15 min	95°C	1 cycle
30 sec	95°C	20 cycles
2 min	65°C	
25 sec	95°C	30 cycles
40 sec	50°C	
40 sec	70°C	
8 min	70°C	1 cycle
∞	4°C	

Cultivated samples

15 min	95°C	1 cycle
30 sec	95°C	10 cycles
2 min	65°C	
25 sec	95°C	20 cycles
40 sec	50°C	
40 sec	70°C	
8 min	70°C	1 cycle
∞	4°C	

Table 1. PCR cycling conditions

Amplification products can be stored at +4°C to -20°C

3. Optional electrophoresis

- For checking the amplification reaction, 5µl of each sample may be directly applied to a 2% agarose gel without the addition of loading buffer.
- Respective sizes of amplification products MTBDR*plus* kit (Table 2).

Gene	Sizes of amplification products (bp)
Amplification control	63
<i>M.tuberculosis</i> complex	115
<i>rpoB</i>	166
<i>katG</i>	20
<i>inhA</i>	110

Table 2. Size of amplification products (MTBDR*plus*)

4. Hybridization

Hybridization should be conducted in a separated area « black room ».

It includes the following steps: chemical denaturation of the amplification products, hybridization of the single-stranded, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate and an AP mediated staining reaction. A template ensures the easy and fast interpretation of the banding pattern obtained.

1. Material

- 1.5 mL screw cap tube
- 1000µl, 200 µl, 100µl and 20µl pipettes
- Disposable sterile pipette tips with filter
- Sterile distilled water
- Pencil
- Gloves
- Absorbent paper
- Tweezer
- transparent adhesive tape
- Oven or waterbath at 37-45 °C
- Shaking platform/TwinCubator®
- MTBDR*plus* kit

2. Preparation

- Pre-warm shaking water bath/TwinCubator® to 45 °C; the maximum tolerated deviation from the target temperature is +/-1 °C.
- Pre-warm solutions HYB and STR to 37-45 °C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary.
- Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature.
- Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10µl concentrate to 1 ml of the respective buffer.
- Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

3. Hybridization protocol

- 1) Dispense **20 μ l of Denaturation Solution (DEN, blue)** in a corner of each of the wells used.
- 2) Add to the solution **20 μ l of amplified sample**, pipette up and down to mix well and incubate at room temperature for 5 min. Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.
- 3) Carefully add to each well **1ml of pre-warmed Hybridization Buffer (HYB, green)**. Gently shake the tray until the solution has a homogenous color. Take care not to spill solution into the neighboring wells
- 4) Place a strip in each well. The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers, turn over strips that might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps
- 5) Place tray in shaking water bath/TwinCubator® and incubate for **30 min at 45 °C**. Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least 1/3 of its height.
- 6) Completely aspirate Hybridization Buffer
- 7) Add **1 ml of Stringent Wash Solution (STR, red)** to each strip and incubate for **15 min at 45 °C** in shaking water bath/TwinCubator®
- 8) Work at room temperature from this step forward. Completely **remove Stringent Wash Solution**. Pour out Wash Solution in a waste container and remove all remaining fluid. This also applies to all other wash steps.
- 9) Wash each strip once with **1 ml of Rinse Solution (RIN) for 1 minute** on shaking platform/TwinCubator® (pour out RIN after incubation).
- 10) Add **1ml of diluted Conjugate** (see above) to each strip and incubate for **30 min** on shaking platform/TwinCubator®.
- 11) Remove solution by turning the tray upside down and gently striking it on an absorbent paper and wash each strip **twice for 1 minute with 1ml of Rinse Solution (RIN) and once for 1 minute with approx. 1ml of distilled water** (e. g. use wash bottle) on shaking platform/TwinCubator® (pour out solution each time). Make sure to remove any trace of water after the last wash.



- 12) Add **1ml of diluted substrate** (see above) to each strip and incubate protected from light without shaking. Depending on the test conditions (e. g. room temperature), the substrate incubation time can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.



- 13) Stop reaction by briefly rinsing twice with distilled water.
14) Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.
15) Paste strips on the results' paper
16) Store in a place protected from light



5. Evaluation and interpretation of results

1. Procedure

An evaluation sheet is provided with the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. For technical reasons, the distances between single probes on the strips may vary slightly. For an accurate evaluation therefore please use the provided template and align it – separately for each of the three loci – with the respective Locus Control band. Determine the resistance status and note down in the respective column. As a help for interpretation, evaluation examples are given in the subsequent chapter. Not all bands of a strip have to show the same signal strength.

2. Quality Control

In order to control that the test goes smoothly and that reagents are performing well, each strip has 4 control zones:

- **Conjugate Control (CC)** A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.
- **Amplification Control (AC)** When the test is performed correctly, a control amplicon will bind to the Amplification Control zone. If this band is developed, mistakes during extraction and amplification setup and the carry-over of amplification inhibitors can be excluded. In case of a positive test result, the signal of the Amplification Control zone can be weak or even vanish totally. This might be due to competition of the single reactions during amplification. In this case, however, the amplification reaction was performed correctly and the test does not have to be repeated. A missing AC band in case of a negative test result indicates mistakes during amplification set-up, or carry-over of amplification inhibitors. In this case, the test is not valid and the respective sample has to be repeated.
- ***M. tuberculosis* complex (TUB)** This zone hybridizes, as known, with amplicons generated from all members of the *M. tuberculosis* complex. If the TUB zone is negative, the tested bacterium does not belong to the *M. tuberculosis* complex and cannot be evaluated by this test system.
- **Locus Controls (*rpoB*, *katG*, and *inhA* (MTBDR*plus*) *gyrA*, *gyrB*, *rrs* and *eis* (MTBDR*s*)).** The Locus Control zones detect a gene region specific for the respective locus and must always stain positive when the TUB zone has documented the presence of a *M. tuberculosis* strain. If neither the Locus Control probe nor the Wild type or Mutation probes of one of the genes examined are developed, the test cannot be evaluated. The signals of the Locus Control bands may be weak

3. Interpretation of the signals

a) *Wild and mutated probes*

Each studied gene is covered by Wild Type probes (WT) and Mutated probes (MUT) containing the mutations associated with the resistance to the antibiotic.

Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone (AC) are to be considered.

Not all bands of a strip have to show the same signal strength.

- **Wild type probe:** The wild type probes comprise the most important resistance areas of the respective genes. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. This result suggests a sensitivity of the strain tested to the antibiotic. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe resulting in the absence of the wild type probe signal. The absence of a

signal for at least one of the wild type probes hence indicates a mutation in the gene suggesting resistance of the tested strain to the respective antibiotics. Each pattern deviating from the wild type pattern indicates a resistance of the tested strain.

- **Mutation probes** The mutation probes detect some of the most common resistance-mediating mutations. Each pattern deviating from the wild type pattern indicates a resistance of the tested strain.

b) *MTBDRplus* genes

GenoType® *MTBDRplus* is a Line Probe Assay for identification of the *M. tuberculosis* complex and its resistance to Rifampicin and/or Isoniazid (*rpoB*(2, 3), *katG*(4) and *inhA* (5)genes).

rpoB

rpoB gene is examined for detection of resistance to rifampicine. The wild probe comprises the most important resistance region of the *rpoB* gene. Four mutation probes detect some of the most common *rpoB* resistance-mediating mutations. Compared to the other probes, positive signals of the *rpoB* MUT2A and MUT2B mutation probes may show a lower signal strength.

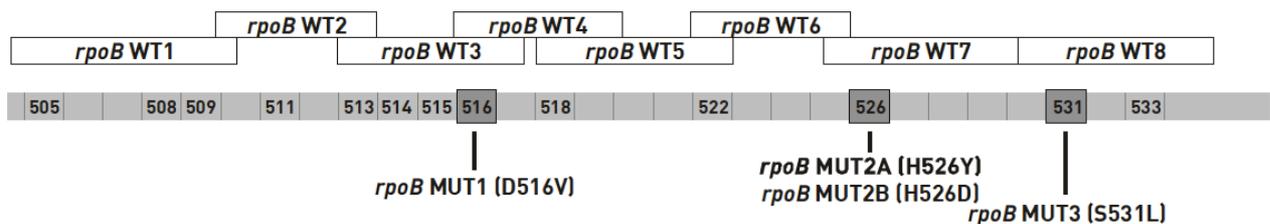


Fig 1. *rpoB* gene 81 bp RMP-resistance determining region and the corresponding wild type and mutation bands

katG

katG gene is examined for detection of high level resistance to isoniazid. Each pattern deviating from the wild type pattern (see evaluation example 2, 4 and 5) indicates high level resistance to INH of the tested strain.

failing wild type band	codon analyzed	developing mutation band	mutation
<i>katG</i> WT	315	<i>katG</i> MUT1	S315T1
		<i>katG</i> MUT2	S315T2

Table 3. Mutations in the *katG* gene and the corresponding wild type and mutation bands

inhA

inhA gene is examined for detection of low level resistance to isoniazid. Each pattern deviating from the wild type pattern (see evaluation example 3) indicates low level resistance to INH of the tested strain.

failing wild type band	analyzed nucleic acid position	developing mutation band	mutation
<i>inhA</i> WT1	-15	<i>inhA</i> MUT1	C15T
	-16	<i>inhA</i> MUT2	A16G
<i>inhA</i> WT2	-8	<i>inhA</i> MUT3A	T8C
		<i>inhA</i> MUT3B	T8A

Table 4. Mutations in the *inhA* promoter region and the corresponding wild type and mutation bands

Example 3: The “*inhA* WT1” band is missing (signal intensity is lower than that of the AC) and the “*inhA* MUT1” band is developed. Accordingly, the field in the “*inhA* WT” column is marked with a “–” and the field in the “*inhA* MUT” column is marked with a “+”. The *rpoB* and *katG* locus show the wild type pattern. Due to the *inhA* result, low level INH-resistance is assigned to the tested strain (see table 4 above).

Example 4: One of the *rpoB* wild type bands is missing and one of the *rpoB* MUT3 mutation band is developed. In the evaluation chart, a “–” is depicted in the field of the “*rpoB* WT” column and the field of the “*rpoB* MUT” column is marked with a “+” and the tested strain is evaluated as RMP-resistant (S531L mutation see Figure 1 above). The *katG* wild type band “*katG* WT” is missing, and the mutation band “*katG* MUT2” is developed; hence, the field in the “*katG* WT” column is marked with a “–”, the field in the “*katG* MUT” column is marked with a “+”. The “*inhA* WT2” band is missing (signal intensity is lower than that of the AC) and the “*inhA* MUT3A” band is developed. Hence, the strain is evaluated as high-level INH-resistant (see table 3 above).

Example 5: From both the *rpoB* and the *katG* locus one wild type bands is missing and none of the *rpoB* and *katG* mutation bands are developed. Therefore, all *rpoB* and *katG* columns are marked with a “–” and RMP-resistance an high level and INH –resistance are assigned to the tested strain. The *inhA* locus shows the wild type banding pattern which is depicted accordingly.

c) *MTBDRsl v 2.0 genes*

gyr A

Both the *gyrA* and *gyrB* genes are examined for detection of resistance to Fluoroquinolones (FLQ) (e.g., ofloxacin or moxifloxacin)(6). Each pattern deviating from the wild type pattern (see evaluation example 1) indicates, as far as is known, resistance to FLQ of the tested strain

Failing wild type band	Developing mutation band	Mutation	Phenotypic resistance
<i>gyrA</i> WT1	-	G88A	FLQ
		G88C	
<i>gyrA</i> WT2	<i>gyrA</i> MUT1	A90V	
	<i>gyrA</i> MUT2	S91P	
	<i>gyrA</i> MUT3A	D94A	
<i>gyrA</i> WT3	<i>gyrA</i> MUT3B	D94N	
		D94Y	
	<i>gyrA</i> MUT3C	D94G	
	<i>gyrA</i> MUT3D	D94H ¹⁾	

¹⁾ rare mutation

Table 5 : Mutations in the *gyrA* gene and the corresponding wild type and mutation bands (according to (7-10))

gyrB

Both the *gyrA* and *gyrB* genes are examined for detection of resistance to FLQ (e.g., ofloxacin or moxifloxacin). The wild type probe comprises the most important resistance region of the *gyrB* gene. The mutation probes detect the most common resistance-mediating mutations (see table 6). Additional mutations within the examined *gyrB* gene region that cause a failing wild type band but are not detected by the mutation probes may also lead to FLQ resistance (11).

Failing wild type band	Developing mutation band	Mutation ¹⁾	Phenotypic resistance
<i>gyrB</i> WT	<i>gyrB</i> MUT1	N538D	FLQ
	<i>gyrB</i> MUT2	E540V	

¹⁾ Amino acid positions are numbered according to (12)

Table 6 : Mutations in the *gyrB* gene and the corresponding wild type and mutation bands (according to (11))

rrs

The *rrs* gene is examined for detection of cross-resistance to AG/CP antibiotics such as kanamycin (KAN) and amikacin (AMK), both AG, or capreomycin (CAP) and viomycin (VIO), both CP. The wild type probes comprise the most important resistance regions of the *rrs* gene. The mutation probes detect the most common resistance-mediating mutations (see table 7). Each pattern deviating from the wild type pattern (see evaluation example 1) indicates, as far as is known, an AG/CP-resistance of the tested strain. The detectable cross-resistances are shown in the table below.

Failing wild type band	Analyzed nucleic acid position	Developing mutation band	Mutation	Phenotypic resistance				See figure 1
<i>rrs</i> WT1	1401	<i>rrs</i> MUT1	A1401G	KAN	AMK	CAP		example 2 and 6
	1402	-	C1402T	KAN		CAP	VIO	example 3
<i>rrs</i> WT2	1484	<i>rrs</i> MUT2	G1484T	KAN	AMK	CAP	VIO	example 4

Table 7 : Mutations in the *rrs* gene, the corresponding wild type and mutation bands, and the resulting cross-resistances (according to (13, 14))

eis

The *eis* gene is examined for detection of a low-level KAN-resistance. The wild type probes comprise the most important resistance regions of the *eis* gene. The mutation probe detects the most common resistance-mediating mutation (see table 8). More mutations within the examined *eis* gene region than those listed in table 8 are known [19]. These mutations that may be causing a failing wild type band but are not detected by the mutation probe may also cause low level KAN-resistance.

Failing wild type band	Developing mutation band	Mutation	Phenotypic resistance
<i>eis</i> WT1	-	G-37T	low-level KAN
<i>eis</i> WT2	<i>eis</i> MUT1	C-14T	
	-	C-12T	
	-	G-10A	
<i>eis</i> WT3	-	C-2A	

Table 8 : Mutations in the *eis* promoter region and the corresponding wild type and mutation bands (according to (15-18))

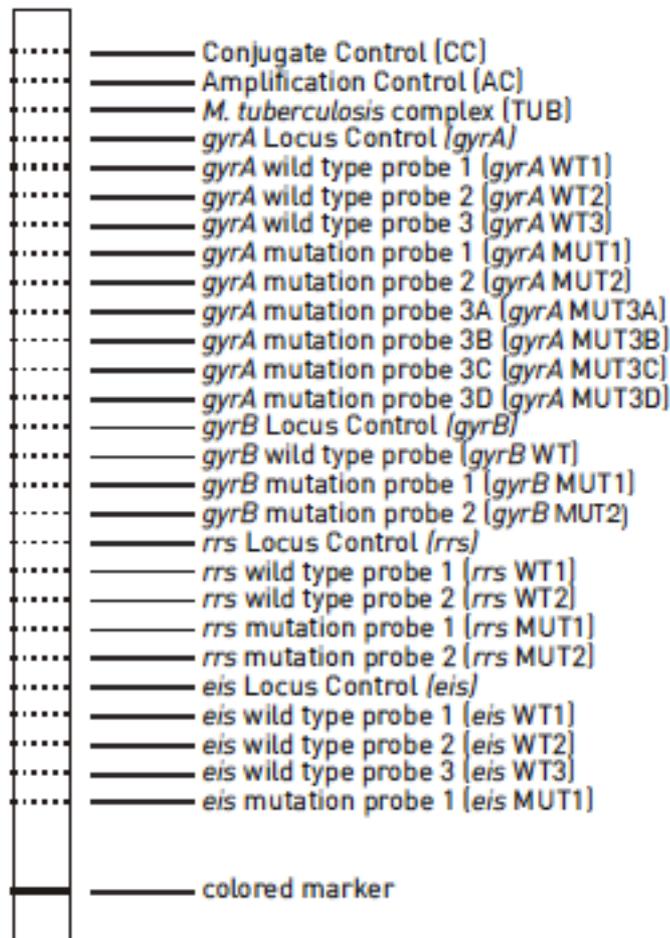


table 7 above). Finally, the probes of the *eis* locus display the wild type banding pattern; hence, the columns “*eis* WT” and “*eis* MUT” are marked according to example 1 and no low-level KAN resistance is detected.

Example 3: The “*gyrA* WT2” band is missing (signal intensity is lower than that of the AC) and the “*gyrA* MUT2” band is developed. Accordingly, the field in the “*gyrA* WT” column is marked with a “–” and the field in the “*gyrA* MUT” column is marked with a “+”. The *gyrB* locus displays the wild type banding pattern which is depicted accordingly. Due to the *gyrA* result, FLQ-resistance is assigned to the tested strain. The *rrs* wild type band “*rrs* WT1” is missing, but none of the *rrs* mutation bands is developed; thus, the fields in the “*rrs* WT” and “*rrs* MUT” columns are marked with a “–” and cross-resistance to KAN, CAP, and VIO is identified (see table 7 above). The *eis* locus displays the wild type banding pattern which is depicted accordingly.

Example 4: One of the *gyrA* wild type bands is missing and one of the *gyrA* mutation bands is developed. In the evaluation chart, a “–” is depicted in the field of the “*gyrA* WT” column and the field of the “*gyrA* MUT” column is marked with a “+”. The *gyrB* locus displays the wild type banding pattern which is depicted accordingly. Due to the *gyrA* result, FLQ-resistance is assigned to the tested strain. The *rrs* wild type band “*rrs* WT2” is missing and the mutation band “*rrs* MUT2” is developed; thus, the field in the “*rrs* WT” column is marked with a “–”, the field in the “*rrs* MUT” column is marked with a “+”, and the tested strain is evaluated as cross-resistant to KAN, AMK, CAP, and VIO (see table 3 above). The *eis* locus displays the wild type banding pattern which is depicted accordingly.

Example 5: From both the *gyrA* and the *gyrB* locus one wild type bands is missing and none of the *gyrA* and *gyrB* mutation bands are developed. Therefore, all *gyrA* and *gyrB* columns are marked with a “–” and FLQ-resistance is assigned to the tested strain. The *rrs* locus shows the wild type banding pattern which is depicted accordingly. Finally, one of the *eis* wild type bands is missing; hence, both the fields in the “*eis* WT” and “*eis* MUT” columns are marked with a “–” and a low-level KAN resistance is detected.

Example 6 shows the wild type banding pattern for the *gyrA* locus which is depicted accordingly. The *gyrB* wild type band is missing and one of the *gyrB* mutation bands is developed. Hence, in the evaluation chart, a “–” is depicted in the field of the “*gyrB* WT” column and a “+” in the field of the “*gyrB* MUT” column. Due to the *gyrB* result, FLQ resistance is assigned to the tested strain. The *rrs* wild type band “*rrs* WT1” is missing and the mutation band “*rrs* MUT1” is developed; hence, the field in the “*rrs* WT” column is marked with a “–”, the field in the “*rrs* MUT” column is marked with a “+”, and the strain is evaluated as cross-resistant to KAN, AMK, and CAP (see table 3 above). One of the *eis* wild type bands is missing and the *eis* mutation band is developed. Hence, in the *eis* WT column, a “–” is depicted, the *eis* MUT column is marked with a “+”, and a low-level KAN resistance is assigned to the tested strain.

Example 7: Both the *gyrA* locus and the *gyrB* locus show the wild type pattern which is depicted accordingly with respect to FLQ resistance. The *rrs* locus shows the wild type pattern which is depicted accordingly. One of the *eis* wild type bands is missing. Hence, a “–“ is depicted in both the “*eis* WT” and the “*eis* MUT” column of the evaluation chart, and a low-level KAN resistance is assigned to the tested strain.

6. Troubleshooting

1. Overall weak or no signals (including Conjugate Control zone)

- Room temperature too low or reagents not equilibrated to room temperature.
- No or too little amount of CON-C and/or SUB-C used.

2. Weak or no signals except for Conjugate Control zone

- Quality and/or quantity of extracted DNA do not allow an efficient amplification. Check amplicon on a 2% agarose gel. In case no amplicon is visible, repeat DNA extraction and amplification. If necessary, try a different DNA extraction method.
- Incubation temperature too high.

3. No homogeneous staining

- Strips were not completely immersed during incubation steps.
- Tray was not shaken properly.

4. High background color

- CON-C and/or SUB-C used too concentrated.
- Washing steps were not performed with the necessary care.
- Wash solutions too cold.

5. Unexpected result

- Wrong incubation temperature.
- Hybridization Buffer and/or Stringent Wash Solution were not properly pre-warmed or mixed.
- Contamination of extracted DNA and/or amplification agents with extracted and/or amplified DNA. In case amplification agents are contaminated a negative control sample also shows the respective banding pattern.
- Contamination of neighboring wells by spillage during addition of Hybridization Buffer.
- Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast color development may occur. In such cases, discontinue the substrate incubation as soon as the signals

are clearly visible in order to prevent the development of cross-hybridizing bands.

- No pure culture as starting material or more than one mutation in the tested strain.
- Silent mutation in probe region

6. Special cases

When both a mutation probe and the corresponding wild type probe of a strip are developed, this represents a valid result. Possible reasons could be:

- The tested specimen contains a heteroresistant strain.
- The tested specimen contains more than one *M. tuberculosis* complex strain (e.g. due to mixed infection of the patient).

Theoretically, a resistance can exist in spite of a wild type pattern. Possible reasons could be:

- The tested specimen contains a strain that has developed a heteroresistance and the resistance is caused by a mutation not covered by the mutation probes.
- The tested specimen contains a wild type and a resistant strain (e.g. due to mixed infection of the patient) and the resistance is caused by a mutation not covered by the mutation probes.

When a complete gene locus (all bands including the Locus Control band) is missing, this is an invalid result. If this result is generated from a clinical specimen, possible reasons could be, but are not limited to, a DNA concentration in the sample below the limit of detection.

7. Limitations

Strictly adhere to the established protocols and procedures in order to obtain correct test results and to avoid contaminations.

Use of this assay is limited to qualified personnel well trained in the test procedure and familiar with molecular biological methods.

The test reflects the current state of knowledge of Hain Lifescience.

As with any DNA-based assay, this test only screens the nucleic acid sequence and not the amino acid sequence. Therefore, it is possible that mutations in the probe region that do not cause an amino acid exchange (silent mutations) will still produce the absence of one of the wild type bands.

The GenoType® MTBDR*plus* test only detects those resistances of the *M. tuberculosis* complex that have their origins in the *rpoB* (2), *katG* (4) and *inhA* (5) regions examined here. Resistances originating from mutations of other genes or gene regions as well as other rifampicin and isoniazid resistance mechanisms will not be detected by this test.

The GenoType MTBDRs/ test only detects those resistances that have their origins in the *gyrA*, *gyrB*, *rrs*, and *eis* gene regions examined here. Resistances originating from mutations of other genes or gene regions as well as other FLQ or AG/CP resistance mechanisms will not be detected by this test. The data given in table 7 regarding the cross-resistances between KAN, AMK, CAP, and VIO reflect the current state of knowledge of Hain Lifescience.

The members of the *M. tuberculosis* complex cannot be differentiated.

The presence of multiple bacterial species in the sample to be analyzed might hamper the interpretation of the test.

As any DNA detection method the test system on hand detects DNA from viable and nonviable bacteria. Therefore, the GenoType tests may not be used for monitoring the progression or success of treatment of patients with antimicrobial therapy.

The GenoType tests generate qualitative results. The intensities of the bands on a strip do not give information about the number of cells in a positive sample.

The results of this test may only be interpreted in combination with additional laboratory and clinical data available to the responsible physician. In addition, results of phenotypic drug susceptibility testing have to be considered in certain cases.

The user must have or acquire information about the local mutation distribution pattern of the genes investigated with this test. Confirmation of the test results by phenotypic drug susceptibility testing may be necessary.

8. Bibliographic references

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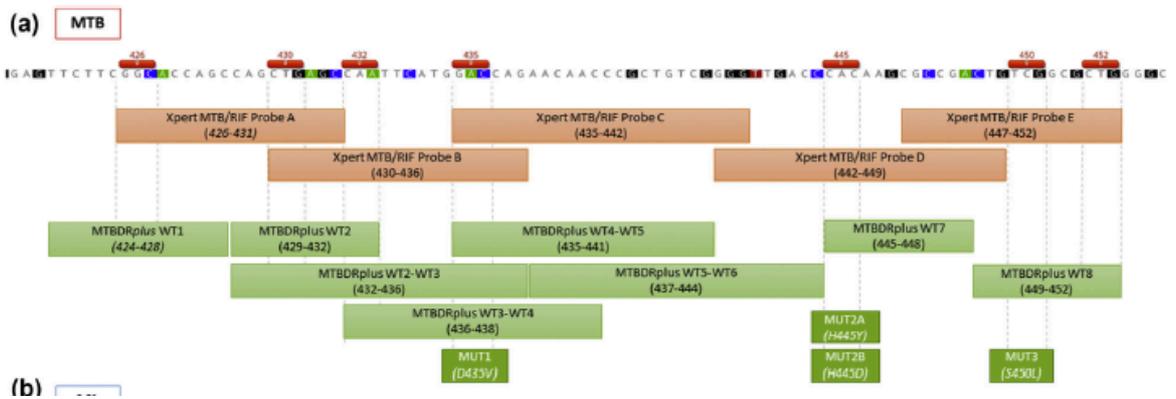
SUPPLEMENT 1

rpoB gene 81 bp RIF-resistance determining region and Xpert MTB/RIF and MTBDR*plus* probes



rpoB gene 81 bp RIF-resistance determining region and Xpert MTB/RIF probes

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